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# Inhibition of Gluconeogenesis in Cultured Chicken Embryo Hepatocytes by *Fusarium* Metabolites

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**ABSTRACT** Four *Fusarium* metabolites, 2,5-anhydro-D-mannitol, 2,5-anhydro-D-sorbitol, moniliformin, and fumonisin $B_1$, were tested for their ability to inhibit gluconeogenesis and cell viability in a primary chicken embryo hepatocyte culture system. The hepatocyte system was established from fertilized chicken eggs that were incubated for 14 days. The hepatocytes produced and secreted glucose into the supernatant of a Krebs incubation solution amended with 3 mM of either lactate or fructose as a precursor for glucose formation. 2,5-Anhydro-D-mannitol and 2,5-anhydro-D-sorbitol inhibited gluconeogenesis in these cells from both lactate and fructose. The former anhydro sugar was more inhibitory when lactate was the precursor (50% inhibition, $IC_{50}$, 6 mM) and the latter anhydro sugar more inhibitory when fructose was the precursor ($IC_{50}$, 12 mM). Moniliformin was more inhibitory to glucose formation from lactate ($IC_{50}$, 100 μM) than from fructose in these cells. The degrees of inhibition of gluconeogenesis by the two anhydro sugars and moniliformin were greater than their effect on cell viability. Fumonisin $B_1$ as high as 1 mM neither inhibited gluconeogenesis, nor affected cell viability. *Nat. Toxins 5:80–85, 1997.* 

**Key Words:** gluconeogenesis; hepatocytes; β-D-fructose analogs; 2,5-anhydro-D-mannitol; 2,5- anhydro-D-sorbitol; moniliformin; fumonisin $B_1$

## INTRODUCTION

Many *Fusarium* species produce a number of secondary metabolites that cause different toxicological responses in plants and animals. These metabolites are grouped as mycotoxins, phytotoxins, antibiotics, and pigments of undefined biological activities [Vesonder et al., 1981]. Many of these metabolites, for example, moniliformin and fumonisins, can be toxic to both plants and animals [Cole et al., 1973; Marasas, 1995]. Further exploration of the biological activities of these metabolites will help us understand better the implications of these compounds in the health and production of plants and animals.

Synthetic 2,5-anhydro-D-mannitol, an analog of β-D-fructose, has been shown to inhibit gluconeogenesis from lactate plus pyruvate in hepatocytes freshly isolated from fasted rats [Riquelme et al., 1983] and to inhibit gluconeogenic carbon flux in tomato fruit pericarp tissue [Halinska and Frenkel, 1991]. Gluconeogenesis has been studied in freshly isolated hepatocytes either from chicken embryos [Dickson, 1983] or posthatched chickens [Schultz and Mistry, 1981]. In this study, we developed a system of cultured primary chicken embryo hepatocytes for observing gluconeogenesis and used this system to test the effects of several *Fusarium* metabolites (2,5-anhydro-D-mannitol, 2,5-anhydro-D-sorbitol, fumonisin $B_1$ and moniliformin) on gluconeogenesis. Fumonisin $B_1$ and moniliformin were tested because they could be produced by the same fusarial strain and they caused lower levels of serum glucose in affected chickens [Jarved et al., 1995].

## MATERIALS AND METHODS

### Chemicals

Fusarial metabolites—namely, moniliformin (MON), fumonisin $B_1$ ($FB_1$) and the two β-D-fructose analogs, 2,5-anhydro-D-mannitol (ADM), 2,5-anhydro-D-sorbitol (ADS)—were isolated and purified from rice individually fermented with *Fusarium proliferatum* (for MON), *F. moniliform* (for $FB_1$), and *F. solani* (for ADM and ADS) by means of duckweed (*Lemma minor L.*) bioassay-directed fractionation [Vesonder et al., 1992]. MON so obtained has similar biological activity of a commercially available MON standard from Sigma Chemical Co. (St. Louis, MO) when simultaneously tested in cultured chicken embryo myocytes [Wu et al., 1995]. Tetrahydrofuran (HPLC grade), the furan core structure of fructose analogs, was purchased from J.T. Baker Chemicals (Phillipsburg, NJ). Structures of these

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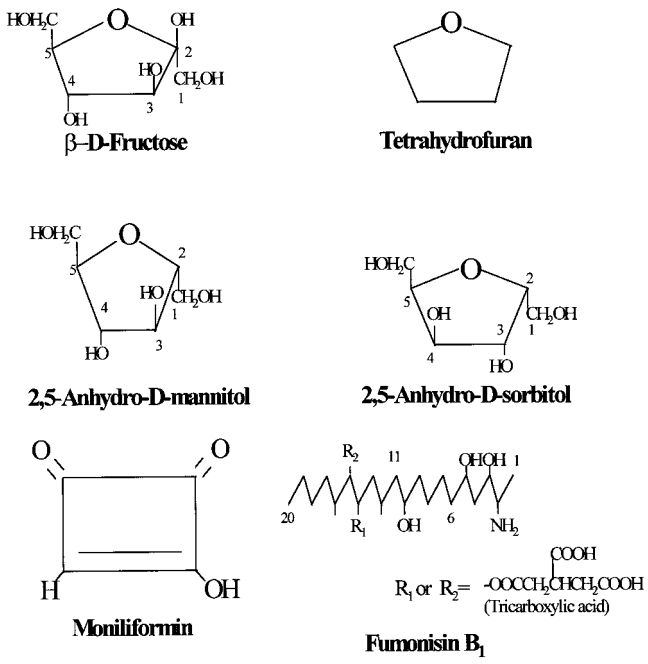


**Fig. 1.** Structure of tested compounds.

tested chemicals are shown in Figure 1. L-Lactate, β-D-fructose, and other chemicals (cell culture-tested), Medium 199, and antibiotic solution (catalog #A-9909), and collagenase (type IV) were all obtained from Sigma Chemical Co. (St. Louis, MO). Newborn calf serum was purchased from Life Technologies, Inc. (Grand Island, NY).

### Isolation and Culture of Primary Chicken Embryo Hepatocytes

Livers were taken aseptically from fertilized Leghorn chicken eggs that had been incubated for 12 to 18 days. The livers were placed in calcium and magnesium free phosphate (0.1 mM) buffered saline (CMF-PBS, pH 7.1)(22°C). After removal of the gall bladders, the liver tissues were transferred to a prewarmed (38.5°C), and modified Krebs solution (NaCl, 121 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; $KH_2PO_4$, 1.2 mM; $NaHCO_3$, 25.5 mM; $CaCl_2$, 1.3 mM) containing antibiotics (100 IU penicillin, 100 μg streptomycin and 0.25 μg amphotericin B/ml), and 0.1% collagenase. The tissues were minced with two pairs of forceps, and then digested with stirring in a water bath (40°C) for 15 min. Then, an equal volume of cold (4°C) unsupplemented Medium 199 was added to the digest, and the mixture poured over four layers of sterile cheese cloth. The freed hepatocytes were collected by centrifugation (500*g* for 5 min), and resuspended in CMF-PBS. Eight milliliters of cell suspension were overlaid atop 2 ml of Histopaque 1077 (Sigma Chemical Co. St. Louis, MO) and centrifuged at 400*g* for 15 min to precipitate the contaminating erythrocytes. Cells at the interface consisting of mainly hepatocytes were transferred to sterile glass test tubes and washed in CMF-PBS twice by centrifugation. The washed cells were resuspended in Medium 199 supplemented with new born calf serum (10%) and antibiotics (100 IU penicillin, 100 μg streptomycin, and 0.25 μg amphotericin B/ml). Concentration of viable cells as determined by trypan blue exclusion was adjusted to $2 \times 10^6$ per ml, and 0.55 ml was placed in each well of a 24-well culture plate (Falcon®, Becton Dickinson Labware, Becton Dickinson & Company, Lincoln Park, NJ). The average number of hepatocytes (including fibroblasts) from each liver of 14-day embryos or 18-day embryos were 8 and 14 million, respectively. Intact viable cells were always higher than 90% in these cell preparations.

### Measurement of Gluconeogenesis

After the seeded plates were incubated overnight in a humidified $CO_2$ incubator (5% $CO_2$ and 95% air) at 38.5°C, the medium was poured off and the wells were rinsed twice with 1 ml of Krebs solution without precursor to remove the exogenous glucose in the medium. Lactate or fructose dissolved in Krebs solution (250 μl) was then fed to the cells along with 50 μl of test compounds in lactate- or fructose-Krebs solution. Each dose was either duplicated or triplicated. A given compound was tested two to five times with different cell preparations. After a given period of incubation, glucose, newly formed from lactate or fructose and released into culture supernatant, was quantified using an enzymatic kit (Stanbio Laboratory, San Antonio, TX, catalog number 1070-125). The assay protocol was modified to a 96-well plate format. Twenty microliters of glucose standards (Stanbio Laboratory, 0 to 20 mg/dl) or culture supernatants were added in duplicate wells of a 96-well flat bottom culture plate (Corning Glass Works, Corning, NY). Then 100 μl of reagent was added to each well with a multiple channel pipette, and mixed for 10 min on a platform fitted on a vortex. The optical density of the red-violet color formed in the plate was measured in a Biotek EL310 Microplate Reader (Biotek Instruments, Inc., Winroski, VT) at 490 nm (the original protocol calls for 500 nm). Intraplate standard curve was established by linear regression. Linearity was observed between 0 and 5 μg ($r^2 = 98$ to 100%) with a detection limit of 0.2 μg. Recovery of a typical supernatant sample added to standards at low, middle, and high end points was 97 ± 5%. All the tested compounds, lactate, and fructose did not interfere with this assay as high as 50 mM in the Krebs solution.

### Measurement of Cell Viability

At the end of incubation, the Krebs solution was removed and 200 μl of cold (4°C) and supplemented Medium 199 containing 0.8 mg/ml MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) was added back. The plates were further incubated for 2 h before the cleavage of MTT to formazan by mitochondrial enzymes was stopped by adding 200 μl of a stopping-lysing solution (20% w/v SDS in N,N-dimethylformamide: water, 50:50; pH adjusted to 4.7).

Complete solubilization of cells and formazan crystals was achieved after the plates were incubated at 37°C overnight. The blue formazan solution (100 μl) was transferred to a 96-well plate and optical density measured at 562 nm in the Microplate Reader. The OD values correlated linearly to the numbers of viable hepatocytes [Wu et al., 1995].

### Statistical Analysis

Percentage of cell viability was calculated as OD of treated wells/OD of control wells × 100. Similarly, percentage of glucose formed was calculated as glucose concentrations in supernatants with inhibitors/concentrations in supernatants without inhibitors (controls) × 100. Percentage was first transformed by arcsine square root (Y/150), where Y = percentage of viability or glucose formed, and then analyzed. The denominator 150 was used to bring the transformed values between the range of 0 to 1. This was necessary because some percentage values were larger than 100% (stimulatory effects). Pooled data from 2 to 5 experiments were subjected to analysis of variance using the GLM procedure of SAS® (SAS Institute Inc., Cary, NC). Preplanned comparison between control and a test dose was made with the LSMEAN procedure after the GLM procedure indicated significant effects of a given tested compound. While transformed data were used in statistical analysis, the original percent values are reported.

## RESULTS

### The Gluconeogenesis System

We first established a set of suitable conditions under which gluconeogenesis in cultured chicken hepatocytes from both glucose precursors, lactate, and fructose, could be easily measured. Hepatocytes from 14-day or 18-day incubated fertile chicken eggs were initially studied. Comparison of time course and level of precursors indicated that hepatocytes from 14-day-old embryos were more effective in gluconeogenesis than the hepatocytes from 18-day-old embryos, and overall lactate was a better precursor for glucose than fructose (Fig. 2). By 2 h of incubation, the hepatocytes from the younger embryos released into the culture supernatants more than 5 mg/dL of glucose for all precursor type and levels, whereas under similar conditions, hepatocytes from the older embryos released less than 5 mg/dl glucose. Less than 0.5 mg/dl of glucose was found in the non-precursor Krebs solution over the 4-h incubation period. All levels of lactate and fructose had no negative effects on cell viability during the 4-h incubation, and the highest level of fructose had the highest cell viability (Fig. 3). It was observed that cells from as early as 12-day-old embryos were capable of producing glucose from either lactate or fructose (data not shown), but the yield of cells from these embryos was not as economical as the older embryos. It was also observed that hepatocytes from 14-day-old embryos retained the capacity of gluconeogenesis after 4 more days of culture in Medium 199, but hepatocytes from

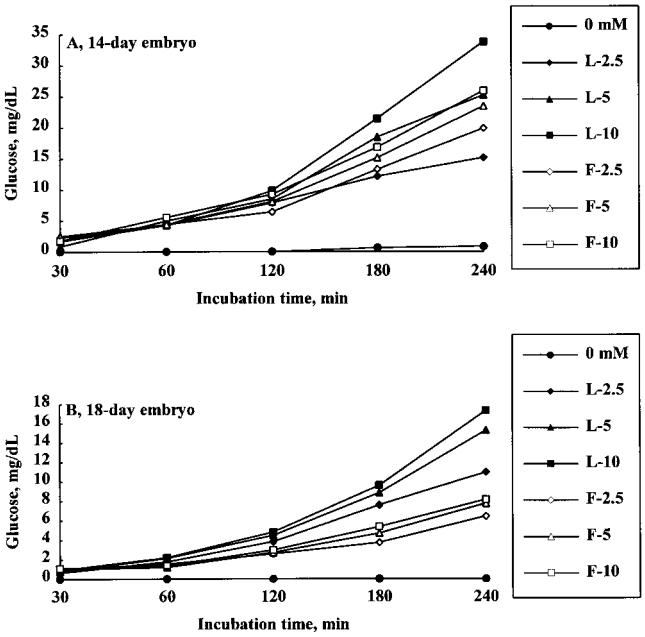


**Fig. 2.** Time course of gluconeogenesis as measured by the accumulation of glucose in the incubation solution supernatant. Hepatocytes isolated from chicken embryos of 14-day **(A)** or 18-day **(B)** incubation were cultured in Medium 199 overnight, washed, and then incubated in Krebs solution containing 0 to 10 mM of either fructose (F) or lactate (L). At the indicated time of incubation, 20 μl of supernatant was removed, and its glucose content measured enzymatically. Glucose concentrations in supernatants after 60 min in all precursor types and levels were higher than those (< 0.5 mg/dl) of 0 mM of precursor ($P < 0.01$). (n = 3, pooled SEM = 0.06 mg/dl).

18-day-old embryos were overpopulated by fibroblasts after 4 more days of culture in vitro, with a concurrent loss of efficiency of gluconeogenesis when compared with cells only cultured overnight (data not shown). It was therefore decided to use hepatocytes isolated from 14-day-old embryos for later experiments. Since the precursors for glucose in the incubation solution were also the only exogenous energy sources, a 2.5 h incubation was chosen to study the potential inhibition of gluconeogenesis by fusarial metabolites while the cytotoxic effects of these compounds due to cellular energy depletion could be minimized. For example, moniliformin is known to inhibit α-ketoacid dehydrogenase complexes [Gathercole et al., 1986]. Concentrations of both precursors for glucose were fixed at 3 mM.

### Effects of β-D-Fructose Analogs

The inhibitory effects of the β-D-fructose analogs on gluconeogenesis are illustrated in Fig. 4. ADM inhibited gluconeogenesis from both lactate and fructose, and more potently if lactate was the precursor, reaching a 50% inhibition at 6 mM (Fig. 4A). Cell viability was significantly ($P < 0.01$) reduced when concentration of ADM was increased above 6 mM, although the reduction was no more

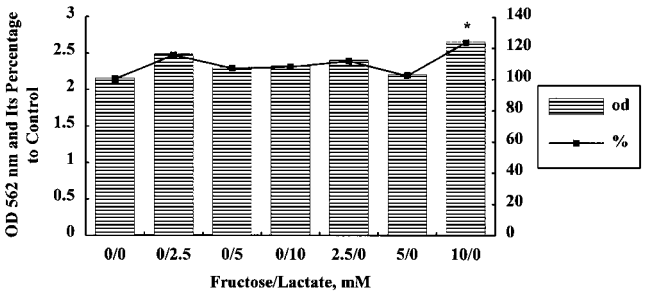


**Fig. 3.** Cell viability of chicken embryo hepatocytes as measured by tetrazolium cleavage assay. In the experiments described in Figure 2, all incubation solutions were removed, and replenished with 200 μl of cold (4°C) Medium 199 containing 0.8 mg/ml of tetrazolium. After 2 h of incubation, cells and formazan crystals were solubilized in acidified SDS-dimethylformamide solution overnight. Formazan was measured spectrophotometrically at 562 nm as an indication of number of living cells. Only 10 mM fructose promoted better viability when compared with 0 mM of precursor (*, $P < 0.05$). This figure represents the observation for cells from 14-day-old embryos. No differences were seen among precursor types and levels for cells from 18-day-old embryos. (n = 3, pooled SEM for OD = 0.097).

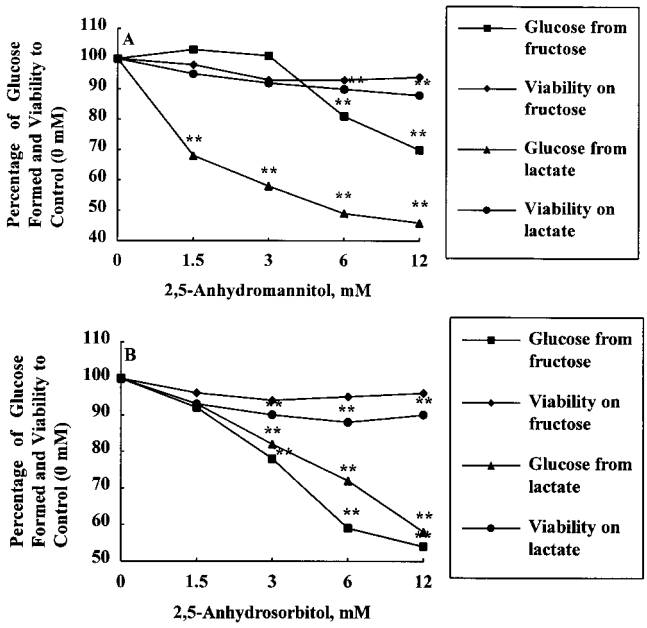


**Fig. 4.** Effects of two β-D-fructose analogs on gluconeogenesis and cell viability. Hepatocytes isolated from 14-day-old chicken embryos were cultured overnight in Medium 199, washed, and incubated in Krebs solution containing 3 mM of either fructose or lactate and varied concentrations of the analogs. Incubation was stopped at 2.5 h. Supernatants were removed, and their glucose contents were measured enzymatically. Cell viability was measured as described in Figure 3. Supernatants of 0 mM precursor contained less than 0.5 mg/dl glucose, and were not subtracted from other supernatants when glucose content was calculated. The points are means of averaged duplicates of five different cell preparations. Significant reductions in glucose formation or viability are indicated by * ($P < 0.05$) or ** ($P < 0.01$). Pooled SEMs for glucose formation and viability from fructose are 4% and 3%, respectively; and from lactate are 6% and 2%, respectively.

than 10%. More than 30% reduction in glucose formation occurred at 1.5 mM of ADM where cell viability was not affected. ADM was a less effective inhibitor and did not affect cell viability if fructose was the precursor, and a 50% inhibition of glucose formation was not observed at concentrations as high as 12 mM. Similarly, ADS also inhibited gluconeogenesis from both lactate and fructose (Fig. 4B). In contrast to ADM, ADS was a more potent inhibitor if fructose was the precursor, with a 50% inhibition at about 12 mM. This inhibition was not associated with the reduction of cell viability. Significant reduction of glucose formation from lactate by ADS was observed at concentrations of 3 mM and higher, along with a significant reduction in viability, although the reduction of viability was no more than 10%. Tetrahydrofuran (core structure for fructose analogs) had no effects on glucose formation from either lactate or fructose nor on cell viability up to 12 mM (data not shown).

### Effects of Moniliformin and Fumonisin $B_1$

Moniliformin at concentrations of 50 μM or higher inhibited glucose formation from both lactate and fructose ($P < 0.01$) (Fig. 5). When lactate served as the precursor, a 50% inhibition was observed at about 100 μM, and significant but less than 10% reduction of viability was associated with MON at 100 and 200 μM. In contrast, when fructose was used as the precursor, MON was a less effective inhibitor and no reduction of cell viability was observed. $FB_1$ as high as 1 mM had no effect on either gluconeogenesis or viability whether lactate or fructose was used as the precursor (data not shown). No levels of $FB_1$ higher than 1 mM were tested because they acidified the bicarbonate-buffered incubation solution.

## DISCUSSION

With a few exceptions where cultured primary ruminant hepatocytes were used in gluconeogenesis studies [Donkin and Armentano, 1993; Lou et al., 1993], a majority of experimental observations reported in the literature were made with fresh hepatocytes immediately after isolation. To our knowledge, this report represents the first use of cultured primary chicken embryo hepatocytes to observe gluconeogenesis. We used overnight culture in the belief that the isolated cells could be allowed to recover from the shock of enzyme digestion and centrifugation, therefore, the cell membrane might resemble more the native state in the tissue. The integrity of the membrane is meaningful in the context of toxicological interpretation of mycotoxin effects. The intact membrane will be less permeable to mycotoxins, particularly the hydrophilic ones. The concentration of ADM required for 50% inhibition of gluconeogenesis from lactate in freshly isolated rat hepatocytes is about 0.1–0.25 mM [Riquelme et al., 1983], whereas in overnight cultured chicken embryo hepatocytes it was 6 mM (this study). This

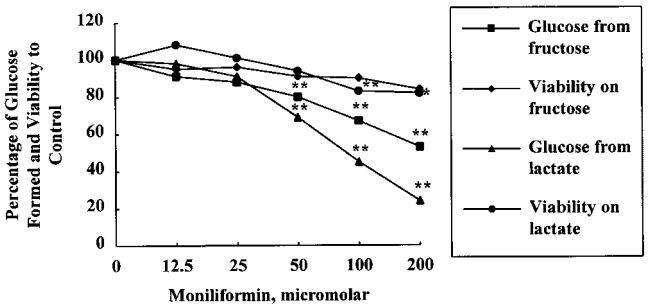


**Fig. 5.** Effects of moniliformin on gluconeogenesis and cell viability. Experimental conditions were same as those for Figure 4. The points are means of duplicates of five different cell preparations. Significant reductions in glucose formation or viability are indicated by *($P < 0.05$) or **($P < 0.01$). Pooled SEMs are for glucose formation and viability from fructose, 9% and 6%, respectively; and from lactate, 8% and 11%, respectively.

discrepancy may reflect the differences in either species or membrane integrity, among many other possibilities.

The effects of ADM in rat hepatocytes are of a dual nature. In cells metabolizing gluconeogenic precursors as triose phosphate (i.e., from lactate and pyruvate), ADM inhibited gluconeogenesis and simultaneously stimulated lactate formation via an activation of pyruvate kinase and inhibition of fructose-1,6-biphosphatase that result from the formation and accumulation of 2,5-anhydro-D-mannitol-1,6-bisphophate [Riquelme et al., 1983]. In cells metabolizing high concentrations of glucose, however, ADM inhibits glycolysis as measured by decreased production of lactate that results from inhibition of phosphoglucose isomerase and phosphofructokinase-1 [Riquelme et al., 1985]. The lack of effect of tetrahydrofuran on gluconeogenesis observed in chicken hepatocytes further supported the formation of a biphosphate analog as an inhibitory mechanism. Phosphofructokinase-1 can not use tetrahydrofuran as a substrate due to the absence of hydroxyl groups. The dual effects of ADM, and perhaps of ADS, may also occur in chicken hepatocytes and merit further investigation. This similar dual effect may not occur with MON since it stimulated glycolysis both in cultured myocytes and hepatocytes as measured by the depletion of exogenous glucose and accumulation of lactate in the culture supernatant (Wu, personal observations). This is the first documentation of inhibition of gluconeogenesis by MON. We are currently investigating the enzymatic and regulatory mechanism of inhibition of gluconeogenesis by MON.

Inhibition of gluconeogenesis by *Fusarium* metabolites could adversely affect the health of both animals and plants. Broiler chicks exposed to $FB_1$, MON, or *F. proliferatum* culture containing these mycotoxins had reduced glucose levels in serum and some died suddenly [Jarved et al., 1995]. Hypoglycemia is associated with the sudden death syndrome of broiler chicken [Brown et al., 1991]. In addition, we have observed that more than 17% symptomless infections of high moisture corn kernels by *F. moniliform, F. proliferatum*, and *F. subglutinans* from farms where the low milk yield in dairy cattle was a concern (Wu, unpublished data). These molds have been shown by scanning electron micrography to be endophytes of corn seeds [Bacon et al., 1992]. It needs to be evaluated whether there is a cause-effect relationship between the inhibitors of gluconeogenesis and animal production and health problems.

2,5-Anhydro-D-mannitol also inhibited gluconeogenesis in plant tissues [Halinska and Frenkel, 1991]. Whether a similar effect of 2,5-anhydro-D-sorbitol occurs in plant cells has yet to be investigated. MON is toxic to both animals and plants [Cole et al., 1973]. Gluconeogenesis is a necessary step during the germination of oil seeds where lipids are mobilized into carbohydrates [ap-Rees, 1980; Li and Ross, 1990]. Many *Fusarium* species, including *F. oxysporum* and *F. solani*, are commonly implicated in damping-offs of agricultural and forest crop seedlings [Bloomberg, 1979; Chakravarty et al., 1990; Garcia and Mitchell, 1975; Odiemah, 1986; Reddy and Rao, 1980; Styer and Cantliffe, 1984]. Some strains of these species are known to produce MON [Marasas et al., 1984] and the fructose analogs [Vesonder, work in progress]. Inhibition of carbon flux from lipids to carbohydrates would impair cell wall formation, which could prevent the emergence of seeds. Whether these compounds are parts of the disease determinant complex of a pathogenic *Fusarium* is both an interesting and important question that needs to be addressed.

## ACKNOWLEDGMENTS

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